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UTILITY  
PATENT APPLICATION  
TRANSMITTAL(Only for new nonprovisional applications under  
37 C.F.R. 1.53(b))

Attorney Docket No.

0050.1609-002

First Named Inventor or  
Application Identifier

Jay M. Edelberg

Express Mail Label No.

EJ611949150US

Title of  
Invention

ENHANCEMENT OF CARDIAC CHRONOTROPY

## APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO:

Assistant Commissioner for Patents  
Box Patent Application  
Washington, D.C. 202311. ☐ Fee Transmittal Form  
(Submit an original, and a duplicate for fee processing)2. ☒ Specification **[Total Pages: 37]**  
(preferred arrangement set forth below)

- Descriptive title of the invention
- Cross References to Related Applications
- Statement Regarding Fed sponsored R & D
- Reference to microfiche Appendix
- Background of the Invention
- Summary of the Invention
- Brief Description of the Drawings
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3. ☒ Drawing(s) (35 U.S.C. 113) **[Total Sheets 5]**  
☒ Formal ☐ Informal4. ☐ Oath or Declaration/POA **[Total Pages [ ]]**

- a. ☐ Newly executed (original or copy)
- b. ☐ Copy from a prior application (37 C.F.R. 1.63(d))  
(for continuation/divisional with Box 17 completed)  
**[NOTE Box 5 below]**
  - i. ☐ **DELETION OF INVENTOR(S)**  
Signed statement attached deleting  
inventor(s) named in the prior  
application, see 37 C.F.R. 1.63(d)(2)  
and 1.33(b).

5. ☒ Incorporation By Reference (useable if Box 4b is checked)  
The entire disclosure of the prior application, from which a  
copy of the oath or declaration is supplied under Box 4b, is  
considered as being part of the disclosure of the accompanying  
application and is hereby incorporated by reference therein.6. ☐ Microfiche Computer Program (Appendix)7. ☐ Nucleotide and/or Amino Acid Sequence Submission  
(if applicable, all necessary)

- a. ☐ Computer Readable Copy
- b. ☐ Paper Copy (identical to computer copy)  
**[ ] Pages**
- c. ☐ Statement verifying identity of above copies

## ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & documents)9. ☐ 37 C.F.R. 3.73(b) Statement ☐ Power of Attorney  
(when there is an assignee)10. ☐ English Translation Document (if applicable)11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS  
Citations12. ☐ Preliminary Amendment13. ☒ Return Receipt Postcard (MPEP 503)  
(Should be specifically itemized)14. ☐ Small Entity Statement(s) ☐ Statement filed in prior application,  
status still proper and desired15. ☐ Certified Copy of Priority Document(s)  
(if foreign priority is claimed)16. ☐ Other: \_\_\_\_\_17. If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information:☒ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No.: PCT/US99/00732

Prior application information: Examiner:

Group Art Unit:

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July 12, 2000

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36,361

DMH/(amg)  
July 12, 2000

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Date: <u>7/12/00</u>	Express Mail Label No. <u>EJ611949150US</u>
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Inventor(s): Jay M. Edelberg and Robert D. Rosenberg

Attorney's Docket No.: 0050.1609-002

## ENHANCEMENT OF CARDIAC CHRONOTROPY

### RELATED APPLICATION

This application is a Continuation of PCT Application No. PCT/US99/00732, filed on January 13, 1999, which claimed priority to U.S. Provisional Application Serial  
5 No. 60/071,456, filed January 13, 1998, entitled "Enhancement of Cardiac Chronotropy", the entire teachings of which are incorporated herein by reference.

### BACKGROUND OF THE INVENTION

The natural pacemaker of the mammalian heart is the sinoatrial node (SA node) which is located in the high right atrium, and which comprises specialized pacemaker  
10 cells that generate electrical impulses characterized by an intrinsic rhythm. The electrical impulse, or pacemaker potential results from the spontaneous depolarization (a bioelectrical process involving the influx and egress of ions which reduces a membrane potential to a less negative value) of the cardiomyocytes within the SA node. This depolarization spreads from the sinus node through the surrounding atrial tissue and  
15 then into the atrial-ventricular node (AV node) before proceeding into the ventricular conduction system. Cardiac chronotropic incompetence resulting from cardiac conduction pathway dysfunction results in abnormalities of the cardiac cycle which are commonly referred to as arrhythmias.

It has been demonstrated that  $\beta$ -adrenergic receptors ( $\beta$ AR) regulate cardiac myocyte  
20 inotropic and chronotropic responses through a G protein-linked signaling pathway (Holmer, S. R., and Homcy, C. J., *Circulation*, 84(5):1891-1902 (1991); Inglese, J. et

*al.*, *J Biol Chem.* 268(32):23735-23738 (1993); Lefkowitz, R. J., and Caron, M. G., *J Biol Chem.* 263(11):4993-6 (1988)). These signaling pathways involve both  $G_{\alpha_s}$ -direct and cAMP-mediated interactions with ion channels involved in myocyte depolarization. Stimulation of  $\beta$ AR increases heart rate as well as cardiac inotropic force. Conversely, blockade of  $\beta$ AR decreases heart rate and cardiac contractility. Cardiac chronotropic incompetence is associated with an increased prevalence of morbidity and mortality.

The majority of the causes of chronotropic incompetence require the implantation of an electronic pacemaker, either temporarily or permanently. The dangers of such surgical procedures are well known. Furthermore, electronic pacemaker devices are subject to failure, which necessitates subsequent surgical procedures to replace the defective device. Future treatments for chronotropic incompetence may be based on therapeutics (biological pacemakers) which can specifically enhance the pacemaker potential of endogenous cardiac tissue. Therefore, it is useful to provide novel compositions and alternative methods to alleviate chronotropic incompetence without the necessity of surgical intervention.

## SUMMARY OF THE INVENTION

The present invention relates to compositions and methods for regulating heart rate comprising localized introduction (e.g., delivery) of one or more exogenous genes to cardiac tissue. Specifically encompassed by the present invention are methods employing gene therapy strategies to provide molecular-mediated or cellular-based biological pacemakers to treat cardiac chronotropic and conduction disorders. The resulting ability to reconstitute the function of defective  $\beta$ -adrenergic signaling cascade in the myocardial tissue localized to the SA node of patients with arrhythmias, cardiac disease, or age-associated myocardial dysfunction offers great hope for the reduction of morbidity and mortality.

As described herein, a murine cDNA chronotropic test system was developed to evaluate the effects of expressing the human  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR) in mice under *in vitro*, *ex vivo* and finally *in vivo* conditions. The ability of  $\beta_2$ AR gene therapy

to restore the normal function of endogenous cardiac tissue was further evaluated in a direct porcine cardiac gene therapy system. More specifically, the present invention describes a gene therapy strategy which utilizes localized expression of biological pacemakers to restore the function of the  $\beta$ -adrenergic signaling cascade. The strategy results in improved cardiac performance and is a useful modality to restore the chronotropic and inotropic responsiveness of dysfunctional or senescent mammalian cardiac tissue.

In one embodiment, the biological cardiac pacemaker is a molecularly-mediated pacemaker. The molecularly-mediated pacemaker is an expression construct comprising at least one gene encoding a cellular protein which either upregulates heart rate, alters cardiac rhythm, or encodes a receptor protein or signal transduction molecule which is essential to normal physiologic cardiac conductance. The gene, or genes, are operably linked to expression control sequences. The expression construct comprising the molecularly-mediated pacemaker can mediate either transient or stable expression. For example, the molecularly-mediated pacemakers can be transiently expressed, and can comprise at least one gene selected from the group consisting of a  $\beta_2$ AR gene, a  $\beta_1$ AR gene, and a  $G_{\alpha s}$  gene. The gene can encode either the endogenous protein or a heterologous protein which is sufficiently homologous to the endogenous protein to possess biological activity in the recipient host cell. In an alternative embodiment, the molecularly-mediated pacemaker can comprise at least one gene selected from the above listed group operably linked to expression control sequences suitable for transient expression under the control of a cardiac tissue specific promoter, which can be either constitutive or inducible. In a further embodiment the cardiac tissue promoter can be specific for atrial tissue.

The invention also pertains to a cellular-based biological cardiac pacemaker utilizing genetically modified cells. A cellular-based cardiac pacemaker can comprise at least one cell transfected or transduced with at least one gene that upregulates heart rate or alters cardiac rhythm, for example a  $\beta_2$ AR gene a  $\beta_1$ AR gene or a  $G_{\alpha s}$  gene.

The invention also encompasses methods of regulating *in vivo* cardiac pacemaking (chronotropic) activity in a mammal by introducing one of the biologic cardiac pacemakers described herein into the SA node region of an endogenous mammalian heart. The mammal for example be a human. The biological pacemaker is introduced into the heart of the mammal, for example, into the right atrium at a site which is localized to a region surrounding the sinoatrial node. The chronotropic method can employ a molecularly-mediated cardiac biological pacemaker comprising at least one gene that upregulates heart rate or alters cardiac rhythm under the control of expression control elements which mediate either transient expression or stable expression, which is either constitutive or inducible.

Cardiac pacemaking activity can also be controlled by a method employing a cellular-based cardiac biological pacemaker comprising at least one myocyte transfected or transduced with at least one gene that upregulates heart rate or alters cardiac rhythm introduced (transplanted or grafted) into the SA node region of the right atria of the recipient host mammal.

The cardiac chronotropy methods described herein can be used for an individual suffering from cardiac conductive tissue incompetence (arrhythmias) indicative of a underlying disorder of cardiac impulse generation, or to treat an older patient experiencing age-related defects in cardiac performance. For example, the method may be useful in clinical conditions characterized by an abnormal sinus rhythm including but not limited to individuals having sick sinus syndrome, sinus bradycardia, or heartblock.

The methods can also be used for permanently regulating cardiac pacemaking activity in a mammal by introducing a stable cellular-based cardiac pacemaker comprising at least one myocyte transfected or transduced with at least one gene that upregulates heart rate or alters cardiac rhythm, or by introducing an molecularly-mediated cardiac pacemaker transcriptionally regulated for stable expression under the control of an inducible promoter.

The invention also encompasses methods of enhancing the basal heart rate of a mammal by delivery into the mammal of a biological pacemaker comprising exogenous

genes which upregulate heart rate or alters cardiac chronotropic or inotropic responsiveness. The invention further encompasses methods of enhancing (upregulating) inotropic responsiveness (cardiac function) of cardiac tissue by utilizing one of the biological cardiac pacemakers described herein to upregulate heart rate or cardiac rhythm.

The chronotropic regulatory methods may further employ the *in vivo* administration of a receptor agonist having a specific cellular affinity for the molecule mediating the chronotropic or inotropic effect. For example, if the activity of the biological pacemaker is based on the expression of  $\beta_2$ AR, the method could further comprise the systemic or local administration of a cardioselective  $\beta$ -adrenergic agent such as isoproterenol.

Future treatments for chronotropic incompetence may obviate the need for mechanical pacemakers, by employing gene therapy strategies to develop therapeutics (biological pacemakers) which can specifically enhance the pacemaker potential of endogenous cardiac tissue. Therefore, it is useful to provide novel compositions and alternative methods are available to alleviate chronotropic incompetence without the necessity of surgical intervention, and the associated risk of mechanical or electronic failure.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B are graphs depicting the results of an experiment showing *in vitro* cardiac myocyte chronotropic recruitment. A. The percentage of cardiac myocytes contracting, in the  $\beta_2$ AR transfected cells (black boxes) and control cells (white circles), in the presence of increasing concentrations of isoproterenol (0 -  $10^{-3}$  M). B. The percentage of cardiac myocytes with a chronotropic rate greater than 60 bpm, in the  $\beta_2$ AR transfected (black boxes) and control cells (white circles), in the presence of increasing concentrations of isoproterenol (0 -  $10^{-3}$  M).

Figure 2 is a graph showing *in vitro* murine cardiomyocyte chronotropic rates. The average chronotropic rate of  $\beta_2$ AR transfected cardiac myocytes (black bars) and

control cells (white bars) in the presence and absence (baseline) of  $10^{-3}$  M isoproterenol.  $*p < .00001$ ;  $** p < 0.001$ ;  $*** p < 0.05$ .

Figure 3A displays representative ECG tracings recorded from transplanted neonatal murine hearts pre- and two days postinjection with either the  $\beta_2$ AR construct (n = 10) or the control construct (n = 10). Figure 3B is a graph displaying the average heart rate of the transplanted hearts pre- and two days postinjection with the  $\beta_2$ AR construct (white bars) or the control construct (black bars).  $* p < 0.001$   $** p < 0.05$ .

Figure 4A displays representative ECG tracings of endogenous murine hearts pre- and two days post *in vivo* injection with either the  $\beta_2$ AR construct (n = 7) or the control construct (n = 8). Figure 4B is a graph displaying the average heart rate of murine hearts pre- and two days post injection with the  $\beta_2$ AR construct (black bars) or the control construct (white bars). Bar 70  $\mu$ m.  $* p < 0.01$   $** p < 0.05$

Figure 5 A are representative surface ECGs recorded 48 hrs after the intracardiac injection of either a control construct (encoding GFP) or a construct encoding  $\beta_2$ AR. Figure 5 B is a graph summarizing the average cycle lengths pre- and two days post-injection with the control construct (white bars) or the  $\beta_2$ AR construct (black bars).

## DETAILED DESCRIPTION OF THE INVENTION

The physiologic depolarization of the heart originates in the sinus node located in the high right atrium. This depolarization spreads from the sinus node through the surrounding atrial tissue and then into the atrial-ventricular node before proceeding into the ventricular conduction system. The rate of sinus node depolarization results from the spontaneous depolarization of myocytes within the node (DiFrancesco, D., *Nature*, 324(6096):470-473 (1986)). These spontaneous cellular depolarizations are automatic, and are, in turn, subject to both sympathetic and parasympathetic regulation. Myocytes from other areas of the heart also depolarize spontaneously, but at physiologic frequencies significantly lower than those of sinus nodal myocytes. Thus, impulses originating from sinus node depolarization suppress the spontaneous activity of

myocytes in other areas. Increased activation of the sinus node elevates heart rate, whereas depressed activation of sinus node may lead to cardiac activation by impulses originating from other areas of the heart.

$\beta$ -adrenergic receptors belong to a large family of G-protein coupled receptors characterized by a homologous structure which includes seven transmembrane domains. Three isoforms of  $\beta$ -adrenergic receptors, designated  $\beta_1$ -  $\beta_2$ - and  $\beta_3$ -adrenoreceptors have been cloned from mammalian tissue (Hajjar, R.J. *et al.*, *J. Mol. Med.* 76: 747-755 (1998)). The cardiac  $\beta$ -adrenoreceptor signaling pathway is made up of the  $\beta_1$ - and  $\beta_2$ -adrenoreceptor, which are coexpressed in the myocardium. Extensive previous research has demonstrated that  $\beta$ -adrenergic receptors ( $\beta$ AR) regulate cardiac myocyte inotropic and chronotropic responses through a G protein-linked signaling pathway (Holmer, S. R., and Homcy, C. J., *Circulation*, 84(5):1891-1902 (1991); Inglese, J. *et al.*, *J Biol Chem.* 268(32):23735-23738 (1993); Lefkowitz, R. J., and Caron, M. G., *J Biol Chem.* 263(11):4993-6 (1988)). The  $\beta$ -adrenergic receptor ( $\beta$ AR) system plays a major role in cardiac contraction. These signaling pathways involve both  $G_{\alpha_s}$ -direct and cAMP-mediated interactions with ion channels involved in myocyte depolarization. Agonist-mediated stimulation of the  $\beta$ AR activates adenylyl cyclase and triggers the production of cyclic adenosine-3' 5' monophosphate. Stimulation of  $\beta$ AR increases heart rate as well as cardiac inotropic force. Conversely, blockade of  $\beta$ AR decreases heart rate and cardiac contractility. The  $\beta$ AR-regulated response is also seen in cultured cardiac myocytes which exhibit an increased spontaneous depolarization rate as well as an augmented contractile force.

The period of automatic depolarization of the heart is shortened by stimulation of  $\beta$ AR in part through an increase in the flux of diastolic depolarization current ( $I_f$ ) in cardiac myocytes (Guth, B. D., and Dietze, T., *Basic Res Cardiol.* 90(3):192-202 (1995)). Moreover, the sinus node has a higher density of  $\beta$ AR compared with the surrounding atrium (Beau, S. L. *et al.*, *Circ Res.* 77(5):957-963 (1995); Saito, K. *et al.*, *Neurosci Lett.* 96(1):35-41 (1989)), which in turn has a higher  $\beta$ AR density than the rest of the heart (Golf, S. *et al.*, *Cardiovasc Res.* 19(10):636-641 (1985). The



atrioventricular conduction system also has a higher density of  $\beta_2$ -adrenoreceptors compared with the rest of the myocardium (Molenarr, P. *et al.*, *Clin. Exp. Pharmacol. Physiol.* 16(6): 529-533 (1989). The expression pattern of  $\beta$ ARs, and the regulation of their  $I_f$  current, suggest that increasing the density of  $\beta$ ARs expressed in the vicinity of the sinus node will mediate an increase in heart rate.

Heart failure is often characterized by a markedly reduced responsiveness of the  $\beta$ -adrenergic receptor-dependent signaling system and a decreased positive inotropic responsiveness (Lohse, M.J. *et al.*, *Basic Res. Cardiol.* 91(Suppl. 2): 29-34 (1996)). Abnormalities in the  $\beta$ -adrenergic signaling cascade have been associated with impaired inotropic responsiveness in patients with congestive heart failure (CHF) (Drazner, M.H. *et al.*, *Proc. Assoc. Am. Physicians* 109(3) 220-227 (1997)), and patients with cardiac valve disease (CVD) frequently have CHF associated with chronic myocardial  $\beta$ AR desensitization due to persistent exposure to increased circulatory catecholamine levels (Gerhardt, M.A. *et al.*, *Circulation* 98(Suppl): II 275-281 (1998)). In addition, quantitative changes in the expression of genes encoding  $\beta$ AR subtypes in the sinoatrial area of the senescent (aging) heart has been postulated as a determinant of age-associated modifications in heart rate variability and diminished contractile response (Xiao, R.P. *et al.*, *J. Clin. Invest.* 101(6): 1273-1282 (1998); Hardouin, S. *et al.*, *Mech. Ageing Dev.* 100 (3): 277-297 (1998)). Recent studies investigating the role of the  $\beta$ -adrenergic signaling cascade in the aging heart report a nonselective downregulation of both  $\beta_1$ - and  $\beta_2$ -adrenoreceptor isoforms leading to a proportional decrease in receptor density with age which is in turn accompanied by a decreased sensitivity to noradrenaline (Xiao, R. P. *et al.*, *J.Clin. Invest.* 101: 1273-1282 (1998)). This data suggest that in the aging heart, decreased  $\beta$ AR density may be of primary import in the decline of positive inotropic responsiveness. In the human heart, the  $\beta$ -adrenergic receptor-G-protein adenylyl cyclase system is possibly the most powerful physiological mechanism available to acutely increase either heart rate and/or contractility (Brodde, O.E., *Basic Res. Cardiol.* 91 Suppl. 2:35-40 (1996)).

The  $\beta$ ARs are excellent targets for directed localized gene expression in the mammalian heart. The human  $\beta_2$ AR is a particularly attractive target for expression in murine model systems, as it is immunologically distinct from, yet structurally and functionally similar to the murine receptor (Moxham, C. P. *et al.*, *J Biol Chem.*

- 5 261(31):14562-14570 (1986); Chung, F. Z. *et al.*, *FEBS Lett.* 211(2):200-206 (1987); Savarese, T. M., and Fraser, C.M., *Biochem J.* 283 (Pt 1):1-19 (1992)). Transgenic mice constructed with the  $\alpha$ -MHC promoter fused to human  $\beta_2$ AR result in mice overexpressing  $\beta_2$ AR throughout the heart (Milano, C. A. *et al.*, *Science*, 264(5158):582-6 (1994)). These transgenic mice manifest enhanced myocardial
- 10 function with increased heart rate and cardiac inotropy. A transgenic based chronotropism model is limited by the practical limitation that a  $\beta_2$ AR-mediated increase in cardiac chronotropy cannot be separated from an inotropic effect. Therefore, it is difficult to utilize a transgenic model to determine whether a local gene expression strategy designed to increase site-specific  $\beta$ AR density expression in the sinoatrial
- 15 nodal tissue can selectively increase cardiac rate in the intact heart. The transgenic approach as an intermediate step in the evaluation of candidate genes is also significantly limited by the time period necessary for *in vivo* generation of the mice.

The Examples presented herein demonstrate that local elevation of  $\beta_2$ AR density in the right atrium by direct gene transfer increases the rate of the heart (a chronotropic

20 effect). A model evaluation system comprising assays of increasing complexity to assess other candidate genes under *in vitro*, *ex vivo*, and finally *in vivo* conditions was established. This integrated approach permits the prolongation/ optimization of the effect of transferred exogenous genes on basal heart rate and cardiac rhythm, which will be helpful in most contemplated practical usages.

25 The Examples provided herein provide a combination of *in vitro*, *ex vivo*, and *in vivo* gene transfer techniques useful for the to identification and characterization of genes that could be employed to selectively upregulate heart rate and alter cardiac rhythm in an intact heart. The data presented herein demonstrate that the local delivery

of expression constructs and/or molecularly engineered cells can increase cardiac pacemaking activity for varying periods of time.

Previous investigations have developed a variety of approaches for expressing exogenous genes in cardiac myocytes under *in vitro* and *in vivo* conditions. Transient  
 5 transfection of isolated cells by the calcium phosphate or lipofection technique with the subsequent determination of the rate of contraction allows initial screening of candidate genes which might upregulate cardiac rate. Cardiac myocytes were derived from fetal hearts in order to take advantage of their enhanced viability and transfection frequency relative to adult-derived cells (Gustafson, T. A. *et al.*, *Proc Natl Acad Sci U S A*,  
 10 84(10):3122-6 (1987)). However, the efficiency of transfection is relatively low (3%-5%) which requires cotransfection with markers that permit selective monitoring of transfected cells. In Example 1, green fluorescent protein(GFP was utilized in conjunction with inverted microscopy and epifluorescence filters to manually count the contraction rates of different classes of myocytes (spontaneously beating or fast beating  
 15 cells) or a video edge motion detector to obtain the average rate of contraction of all transfected cells. Prior studies have also utilized an adenoviral system to achieve virtually complete transfection of isolated myocytes which obviates the need for marking transfected cells (Kohout, T. A. *et al.*, *Circ Res.*, 78(6):971-7 (1996)). This latter approach has been used to overexpress the human  $\beta_2$ AR in rabbit cardiac  
 20 myocytes with a documented enhancement in the relevant signaling pathway (Drazner, M. H. *et al.*, *J Clin Invest.*, 99(2):288-96 (1997)). In similar fashion, Johns and coworkers transfected cultured rat cardiac myocytes have been transfected with an adenoviral-like vector to express a voltage-gated potassium channel (Johns, D. C. *et al.*, *J Clin Invest.*, 96(2):1152-8 (1995)). This technique represents a powerful approach for  
 25 *in vitro* assessment of candidate genes that upregulate heart rate or alter cardiac rhythm.

Atrial targeting of a transgene may be achieved with previously described atrial specific promoters (Field, L. J., *Science*, 239(4843):1029-33 (1988)), and has been employed to overexpress the human beta-1 adrenergic receptor (Bertin, B. *et al.*,

*Cardiovasc Res.*, 27(9):1606-12 (1993)). For example, the atrial specific atrial natriuretic factor promoter was used in the first reported murine transgenic overexpression model of human  $\beta_1$ AR (Bertin, B. *et al.*, *Cardiovasc Res.*, 27(9):1606-12 (1993)). Interestingly, the initial data from these mice, unlike the data from the  $\beta_2$ AR transgenic mice, indicated no enhanced chronotropy. This effect was potentially due to pronounced down-regulation of the constitutively overexpressed receptor. Atria derived from these mice were subsequently found to possess enhanced basal function and reduced heart rate variability (Mansier, P. *et al.*, *Am. J. Physiol.* 270: 1465-1472 (1996).

Employing inducible elements in concert with the atrial specific promoter may decrease such down-regulation, and may be valuable in the final evaluation of candidate genes, particularly if their expression could specifically be targeted to the sinus node or other critical conduction tissue. Ye *et al.*, have recently reported the regulated (rapamycin-inducible) delivery of a therapeutic recombinant protein after *in vivo* somatic cell gene transfer (Ye, X. *et al.*, *Science* 283: 88-91 (1999)). Delivery strategies such as these facilitate the stable transduction of cells and allow for the selective induction of the transgene by pharmacologic means.

As used herein the term "pacemaker" connotes an object or substance that influences the rate at which a particular phenomenon occurs. Here the relevant phenomenon is the depolarization of the sinoatrial node. As used herein the term "heart rate" refers to the number of heart beats per minute, and "heart rhythm" or "rhythm" refers to the regularity of the heart beat. The term "chronotropy" refers to the speed of impulse (electrical signal resulting from sinoatrial node depolarization) formation. The term "inotropy" refers to the force of cardiac contraction. The term "chronotropism" describes the act or process of affecting the regularity of the heart beat (or heart rate).

In one embodiment, the biological cardiac pacemaker is a molecularly-mediated pacemaker. The molecularly-mediated pacemaker is an expression construct comprising at least one gene encoding a cellular protein which either upregulates heart rate, alters cardiac rhythm, or encodes a receptor protein or signal transduction molecule

which is essential to normal physiologic cardiac conductance, operably linked to expression control sequences. The expression control sequences are capable of directing expression in mammalian cells, for example in human cells. The expression construct comprising the molecularly-mediated pacemaker can be mediate either

5 transient or stable expression. The promoters useful in constructing expression vectors useful as molecularly-mediated cardiac pacemakers may be direct either constitutive or inducible expression. A constitutive promoter directs expression under all conditions of cell growth. An inducible promoter directs expression only in the presence of an inducing agent. For example, a molecularly-mediated pacemaker can be transiently

10 expressed, and can comprise at least one gene selected from the group consisting of a  $\beta_2$ AR gene, a  $\beta_1$ AR gene, or a  $G_{\alpha s}$  gene. The gene can encode either an endogenous protein or a heterologous protein that is sufficiently homologous to possess biological activity in the recipient host cell. In an alternative embodiment the molecularly-mediated pacemaker can comprise at least one gene selected from the above-identified

15 group operably linked to expression control sequences suitable for transient expression under the control of a cardiac tissue specific promoter, which can be either constitutive or inducible. In a further embodiment the cardiac tissue promoter can be specific for atrial tissue.

The use of replication-deficient adenoviral viral vectors allows for a rapid

20 assessment of candidate genes under *in vivo* conditions. This approach has been used with high efficiency for transduction of exogenous genes into intact or transplanted hearts (Kass-Eisler, A. *et al.*, *Proc Natl Acad Sci U S A*, 90(24):11498-502 (1993); Lee, J. *et al.*, *J Thorac Cardiovasc Surg.*, 111:246-52 (1996). However, similar to the transgenic approach, the cardiac expression of adenoviral-mediated genes is widespread

25 and cannot be employed to evaluate local effects of targeted constructs or engineered cells. The *in vivo* delivery of expression vectors by direct injection or lipofection has recently been extended to intact or transplanted hearts which allows local delivery of exogenous genes to the intact organ. The direct introduction of DNA with these methods has successfully modified the function of the heart *in vivo*, although the latter

data have not always completely agreed with *in vitro* results (Kitsis, R. N. *et al.*, *Proc Natl Acad Sci U S A*, 88(10):4138-42 (1991); Gal, D. *et al.*, *Lab Invest.*, 68(1):18-25 (1993); Kitsis, R. N. and L. A. Leinwand, *Gene Expr.*, 2(4):313-8 (1992)). Similarly, intravascular transfection of DNA as outlined above has also been employed to achieve  
5 local coronary arterial expression in native as well as transplanted hearts (Giordano, F. J. *et al.*, *Nat Med.*, 2(5):534-9 (1996)). The discrepancies observed between *in vivo* data and *in vitro* results may be due to low level expression of exogenous genes in restricted areas of the heart.

The invention also pertains to a cellular-based biological cardiac pacemaker  
10 utilizing genetically modified cells. A cellular-based cardiac pacemaker can comprise at least one cell transfected or transduced with at least one gene that upregulates heart rate or alters cardiac rhythm, for example a  $\beta_2$ AR gene a  $\beta_1$ AR gene or a  $G_{\alpha s}$  gene. Transfection refers to the acquisition by a cell of new genetic material (nucleic acid molecules) originating from an exogenous source. Transfection is usually mediated by  
15 physical or chemical means and useful protocols include, but are not limited to DEAE-dextran mediated transfection, DNA coprecipitation, electroporation, naked plasmid adsorption and liposome-mediated transfection. Transduction refers to the process of transferring nucleic acids into a cell using a DNA or RNA viral vector. Suitable viral vectors include, but are not limited to retroviral vectors and replication-deficient  
20 adenovirus vectors. Transfection or transduction of the cells with an expression construct as described above for the molecularly-mediated embodiments of the invention can be accomplished by a variety of techniques which are well known to one of skill in the art. The invention further encompasses methods of regulating *in vivo* cardiac pacemaking (chronotropic) activity in an animal by introducing one of the  
25 biologic cardiac pacemakers described herein into the SA node region of an endogenous mammalian heart. The mammal, can be for example a human. The biological pacemaker is introduced into the heart, for example, into the right atrium localized to a region surrounding the sinoatrial node. The biological pacemaker composition is preferably delivered in a pharmaceutical composition comprising, for example, the

molecularly-mediated expression vector in a volume of phosphate buffered saline with 5% sucrose. A therapeutically effective amount of the biological pacemaker is delivered to a site-specific location (e.g. an area in the upper portion of the right atria). A therapeutically effect amount is that amount which corrects or improves the

5 chronotropic or inotropic defect which characterizes the recipient tissue. The therapeutically effective amount can be delivered preferably in a single administration, although multiple dose are also contemplated.

The chronotropic method can employ a molecularly-mediated cardiac biological pacemaker comprising at least one gene that upregulates heart rate or alters cardiac

10 rhythm under the control of expression control elements which mediate transient expression. The biological pacemaker (e.g., cDNA, an expression vector or genetically-manipulated cells) can be directly injected into the myocardium in the generalized region of the sinoatrial node via a transthoracic or mini-thoracotomy procedure, or may be delivered by using a electrophysiology recording catheter modified for endocardial

15 transfection of the cardiomyocytes located in the vicinity of the sinoatrial node.

*In vivo* transfection (gene transfer) into cardiac tissue can be achieved by direct intracardiac injection of plasmid DNA, or may occur pursuant to a virus-mediated gene transfer protocol (e.g. transduction) using for example modified adenoviral vectors, or hemagglutinating virus of Japan (HJV)/liposome-mediated gene transfer. HJV-

20 liposome-mediated transfer, like adenovirus-mediated protocols do not require cell replication and thus can be used to genetically modify terminally differentiated cells such as cardiomyocytes (Ellison, K. E. *et al.*, *J. Mol. Cell. Cardiol.* 28: 1385-1399 (1996)).

Alternatively, site-specific gene delivery may be achieved using a controlled-

25 release delivery method such as that reported by Labhasetwar *et al.*, who describe the development of a proprietary DNA polymer solution which can be used to coat medical devices such as sutures, stents or catheters (Labhasetwar *et al.*, *J. Pharm. Sci.* 87(11): 1347-1350 (1998)).

Site-specific delivery of a biological pacemaker described herein can also be accomplished using an electrophysiologically guided technique to identify the particular portion of the right atria where the sinoatrial node resides. One skilled in the art will be readily familiar with established cardiac mapping techniques thereby enabling them to deliver a biological cardiac pacemaker to the sinoatrial region of the right atrial chamber.

The invention also pertains to a cellular-based biological cardiac pacemaker utilizing genetically modified cells (transplanted or grafted) into the SA node region of the right atria of the recipient host mammal. A cellular-based cardiac pacemaker can comprise at least one cell transfected or transduced with at least one gene that upregulates heart rate or alters cardiac rhythm, for example a  $\beta_2$ AR gene a  $\beta_1$ AR gene or a  $G_{\alpha_s}$  gene. Transfection or transduction of the cells with an expression construct as described above for the molecularly-mediated embodiments of the invention can be accomplished by a variety of techniques which are well known to one of skill in the art. Such methods include but are not limited to transfection, adenoviral-mediated or herpes virus vector-mediated gene transfer and fusigenic liposome-mediated DNA transfer. The cells can be cardiomyocytes isolated from fetal or embryonic tissue. Alternatively the cells could be isogenic cells (e.g. cardiomyocytes, myoblasts, skeletal myocotes), cells of allogeneic cardiac-derived cell line, genetically modified skeletal myoblasts, or allogeneic or xenogenic cells which may or may not have been genetically modified to be histocompatible with the recipient host animal.

The cardiac chronotropy compositions and methods described herein can be used for an individual suffering from cardiac conductive tissue incompetence (arrhythmias) indicative of a underlying disorder of cardiac impulse generation, or to treat an older patient experiencing age-related defects in cardiac performance. For example, the method may be useful in clinical conditions characterized by an abnormal sinus rhythm including but not limited to individuals having sick sinus syndrome, sinus bradycardia, or heartblock. The method may also be useful in treating cardiac conductive disturbances responsible for atrial fibrillation, to the extent that the



technique can establish a dominant alternative foci of automatic activity capable of reproducing the normal function of the sinoatrial node. Atrial fibrillation results from disorganized electrical activity in the atria. The disclosed chronotropic methods may also find utility in an individual experiencing a heart attack or transient depression of heart rate.

The chronotropic compositions and methods of the present invention can also be used for permanently regulating cardiac pacemaking activity in an animal by introducing a stable cellular-based cardiac pacemaker comprising at least one myocyte transfected or transduced with at least one gene that upregulates heart rate or alters cardiac rhythm, or by introducing a molecularly-mediated cardiac pacemaker which is transcriptionally regulated under the control of an inducible promoter. The feasibility of this approach is consistent with the observation that the targeted expression of transforming growth factor  $\beta$ -1 by the delivery of intracardiac grafts comprising genetically modified skeletal myoblasts, has been shown to effect the local, long-term delivery of a recombinant molecule to the heart (Koh, G.Y. *et al.*, *J. Clin. Invest.* 95(1):114-121 (1995)). Viable syngeneic grafts were observed as long as three months after implantation, and immunohistochemical analysis confirmed the presence of grafted cells stably expressing TGF- $\beta$ 1. Furthermore, the ability to establish a stable intracardiac graft in a large species has been demonstrated by studies documenting the successful formation of stable fetal cardiomyocyte grafts in the myocardium of dystrophic dogs (Koh, G.Y. *et al.*, *J. Clin. Invest.* 96(4):2034-2042 (1995)). Engrafted fetal cardiomyocytes, which were identified by dystrophin immunoreactivity, were observed to be tightly juxtaposed with host cardiomyocytes as long as 10 weeks after engraftment.

The invention also encompasses methods of enhancing the basal heart rate of a mammal by delivery into the mammal of a biological pacemaker comprising exogenous genes which upregulate heart rate or alters cardiac chronotropic or inotropic responsiveness. The invention further encompasses methods of enhancing (upregulating) inotropic responsiveness of cardiac tissue by utilizing one of the

biological cardiac pacemakers described herein to upregulate heart rate or cardiac rhythm.

The chronotropic regulatory methods may further employ the *in vivo* administration of a receptor agonist having a specific cellular affinity for the molecule  
5 mediating the chronotropic or inotropic effect. As used herein the term "agonist" means a drug that has an affinity for, and whose binding to, a cell surface receptor, triggers a biochemical response which mediates a physiologic activity. For example, if the activity of the biological pacemaker is based on the expression of  $\beta_2$ AR, the method can further comprise the systemic or local administration of a cardioselective agent,  
10 such as a  $\beta$ -adrenergic agonist, for example isoproterenol.

As described in Example 1, transient transfection of cultured myocytes with expression vectors and lipofectamine was employed as the initial screen for assessing candidate genes that upregulate heart rate or alter cardiac rhythm. In Example 2, a  
15 similar approach was utilized to locally deliver exogenous genes to the intact contracting murine heart transplanted into the mouse ear which permits a rapid appraisal of the action of the candidate gene at the whole organ level that can be used for rapid evaluation of multiple constructs. In Example 3, the exogenous gene was injected into the right atrium of the intact murine heart to determine its effect on heart rate and cardiac rhythm under conditions approaching the situation under which it will be  
20 ultimately utilized. In Example 4, a porcine cardiac gene transfer system was established to evaluate the use of biological pacemakers in the endogenous heart of a large animal. The Yorkshire pig was specifically chosen for these experiments for its anatomic and physiologic similarity to the human cardiovascular system. Moreover, the porcine model has been successfully been employed in gene therapy studies involving  
25 cardiac vasculature. This system also provided an opportunity to develop a transvenous catheter delivery approach that could potentially be employed in human clinical trials.

The expression of the human  $\beta_2$ AR in isolated murine fetal myocytes led to a significant recruitment of cardiac cells to both spontaneously contract as well as to beat at a higher rate. This suggests that the expression of  $\beta_2$ AR leads to both increased

automatic depolarization of myocytes as well as a higher steady state signaling through the receptor. The latter result is in accord with recent studies in which adenoviral-mediated overexpression of human  $\beta_2$ AR in rabbit cardiac myocytes generates raised adenylate cyclase activity (Drazner, M. H. *et al.*, *J Clin Invest.*, 5 99(2):288-96 (1997)). The addition of the adrenergic agonist isoproterenol recruited a higher percentage of control myocytes to the contractile state, but did not alter the percentage of contracting  $\beta_2$ AR transfected cells. Moreover, saturating levels of isoproterenol,  $10^{-3}$  M, raise the same percentage of myocytes to the higher contractile rate in both  $\beta_2$ AR transfected and control myocytes. The concentration of isoproterenol 10 required to achieve maximal stimulation is similar to levels of agonist used in previous studies employing embryonic cardiac myocytes (Barnett, J. V. *et al.*, *J Biol Chem.*, 264(18):10779-86 (1989); Pennock, G. D. *et al.*, *J Pharmacol Exp Ther.*, 268(1):216-23 (1994)). Finally, the above level of agonist increased the average contractile rate of the  $\beta_2$ AR transfected myocytes to significantly higher levels as compared to control cells.

15 These data suggest that enhanced signaling through  $\beta_2$ AR overexpression leads to an increased rate of spontaneous cardiac myocyte beating, as well as an increased extent of steady state signaling which further augments the rate of contraction. These two effects are induced in control cells by addition of isoproterenol, and the employment of the agonist in combination with transfection allows an augmented response to the drug.

20 The *in vitro* experiments outlined above served as an excellent foundation for extending the investigation to the next phase of the evaluation. The results obtained with cultured cardiac myocytes transfected with the  $\beta_2$ AR suggested that expression of the receptor in cardiac tissue should result in an increased heart rate. The above hypothesis was initially tested in an *ex vivo* model, described in Example 2. The

25 transplanted neonatal hearts served as an intermediate test in the progression from *in vitro* to *in vivo* models of  $\beta_2$ AR gene transfer. The subdermally transplanted heart, as compared to the native heart, possesses the advantage of being easily accessible which permits injection of constructs under direct observation without the need for complex surgical procedures. Furthermore, ECGs of the transplanted hearts can be recorded

from leads attached to the host ear, and are electrically isolated from the host heart which can be utilized as a control.

The injection of transplanted hearts with constructs encoding the  $\beta_2$ AR elevated the basal rate of cardiac contraction of the transplanted heart for several days, presumably during the expression of the  $\beta_2$ AR construct with no other additional alterations in the ECG. These results demonstrate that the *in vitro* observations of  $\beta_2$ AR-mediated enhancement of myocyte chronotropy are predicative of the ability of the transferred exogenous gene to increase basal heart rate in the whole organ. The effects of  $\beta_2$ AR on the spontaneous depolarization of myocytes are of particular importance in this regard. Moreover, the above results suggest that injection of candidate genes into transplanted hearts serve as an excellent model for testing cardiac gene therapy targets.

In Example 3,  $\beta_2$ AR constructs were injected into the right atrium of native murine hearts and were observed to generate a marked increase in cardiac rate as compared to control plasmids for several days presumably during the expression of the  $\beta_2$ AR construct. Similar to observations made with the *ex vivo* model, minimal changes were noted in the electrocardiograms of  $\beta_2$ AR transfected hearts except for the increased basal rate. The expression of the encoded human  $\beta_2$ AR is confined to the right atrium of the injected hearts, as demonstrated by immunohistochemical analyses which suggests that  $\beta_2$ AR-enhanced stimulation is initiated in the right atrium and then proceeds through the normal conduction system of the heart.

In Example 4, constructs encoding either the human  $\beta_2$  adrenergic receptor ( $\beta_2$ AR) or green fluorescent protein (GFP) were injected into the right atrium of native Yorkshire pig hearts. The  $\beta_2$ AR construct significantly enhanced chronotropy, as compared to control injections. The average cycle length of the pig heart rate was 567 +/- 100 ms prior to injection. Two days after injection with plasmid encoding the  $\beta_2$ AR the cycle length decreased to 327 +/- 60 ms, as compared to the control cycle length 488 +/- 130 ms ( $p < .03$ ). The difference in cycle length after control injection was not statistically significant ( $p > 0.3$ ). These changes correlated with a 49% increase in the

average heart rate in the  $\beta_2$ AR injected pigs (183 +/- 28 vs. 122 +/- 25 bpm). The increased heart rate was sustained for 1 to 2 days after which the heart rate trended to baseline levels. Sections of the right atrial tissue at the site of injection revealed the presence of GFP. Immunostaining of the sections revealed a colocalization of the human  $\beta_2$ AR in the co-injected with the  $\beta_2$ AR-encoding constructs confirming that the injection of the cDNA constructs resulted in the expression of the encoded genes. These studies further demonstrate that local targeting of gene expression is a feasible modality to regulate the cardiac pacemaking activity.

In summary, these investigations provide an integrated experimental approach for identifying candidate genes and developing local delivery approaches for maximizing and/or prolonging the effects of these candidate genes in upregulating heart rate and altering cardiac rhythm. The results suggest that the above approaches are useful in the development of both molecularly-mediated and cellular-based cardiac pacemakers.

The following examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of the invention. The teachings of all references cited herein are hereby incorporated by reference.

## EXAMPLES

### EXAMPLE 1: TRANSFECTION OF MURINE MYOCYTES

#### 20 PLASMID CONSTRUCTS:

The human  $\beta_2$ AR cDNA was a gift from Dr. Robert J. Leftowitz (Duke University Medical Center). A 2.25 kb Sal1-BamH1 fragment of the human  $\beta_2$ AR cDNA was ligated into a Sal1-BamH1 site 3' to the  $\beta$  actin promoter ( $\beta$ AP) in a pBR322 vector to generate pBR322- $\beta$ AP- $\beta_2$ AR-SV40. In similar fashion, the bacterial  $\beta$ -galactosidase gene (LacZ) was ligated to the  $\beta$ AP in a pBR322 vector and served as a control expression vector. The plasmid pHGF-S65T encoding the green fluorescent protein (GFP) was purchased from Clontech (Palo Alto, CA).

## MYOCYTE HARVEST

Cultured fetal murine myocardial cells were prepared as previously described (Iwaki, K. *et al.*, *J Biol Chem.*, 265(23):13809-17 (1990); Sen, A. *et al.*, *J Clin Invest.*, 82 (4):1333-8 (1988)). Myocytes from ventricles of 17.5 day old B6D2F1 fetal mice were fragmented with a straight-edge razor. The tissue was then digested with 0.5 mg/ml collagenase II (Worthington Biochemical Corp., Freehold, NJ) and 1.0 mg/ml pancreatin (Sigma Chemical Co., St. Louis, MO) in ADS buffer (116 mM NaCl, 20 mM HEPES, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 5.5 mM glucose, 5.4 mM KCl, 0.8 mM MgSO<sub>4</sub>, pH 7.4) at 37°C for 10 min. The cells were centrifuged at 700 g at 4°C for 5 min. The cells were then plated onto 48-well plates (Falcon Labware, Cockeysville, MD) precoated with 1% gelatin or onto 25 mm<sup>2</sup> square coverslips precoated with 1% gelatin and 20 µg/ml laminin at a density of 10<sup>5</sup> cell/ml in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal calf serum (FCS), and Streptomycin 100 µg/ml, and Penicillin (500 µg/mL). The myocytes were grown at 37°C in 5% CO<sub>2</sub>.

## 15 IN VITRO MYOCYTE TRANSFECTION

The transfection of myocytes with lipofection was optimized. Myocytes were plated as described above and grown overnight. The β<sub>2</sub>AR, LacZ, or GFP expression circular vectors (1:2.5 M/M; 0.125 µg total DNA/well) were incubated with Lipofectamine (Life Technologies) (1 µl/well) in Opti-Mem I (Life Technologies) reduced serum medium (12.5 µl/well) for 30 min at 25°C. After incubation, DMEM (100 µl/well) was added. The myocyte cultures were washed with PBS twice, the Lipofectamine-DNA mixtures were added, and the cultures incubated at 37°C. After 4 hours, an equal volume of DMEM with 20% FCS was added to the cultures. The cultures were incubated overnight and the medium was changed to DMEM with 10% FCS.

The myocytes were assayed 48 hr after transfection. Myocytes cotransfected with GFP were identified by inverted microscopy employing epifluorescence filters for FITC<sub>(excitation 405 nm/emission 490 nm)</sub>. Expression of either human β<sub>2</sub>AR or LacZ was confirmed

by immunostaining or X-gal staining, respectively. Immunostaining for the human  $\beta_2$ AR was performed with a rabbit-antihuman  $\beta_2$ AR polyclonal antibody (Santa Cruz Research, Santa Cruz, CA), which does not cross-react with the murine receptor. The myocyte cultures were washed with PBS and blocked with 10% normal serum in PBS  
 5 for 20 min. Samples were then incubated with the primary antibody at 1.0  $\mu$ g/mL in PBS with 1% bovine serum albumin for one hr in a humid chamber at 25°C. The sample was then washed with PBS three times and then incubated with the secondary donkey anti-rabbit Cy3 polyclonal antibody (Jackson ImmunoResearch, West Grove, PA) at a 1:1000 dilution in PBS with 1% bovine serum albumin for one hr in a humid  
 10 chamber at room temperature. The samples were washed with PBS three times and mounted with 90% glycerol in PBS.

#### MYOCYTE CONTRACTION RATE DETERMINATION

The percentage of beating myocytes was determined for cells transfected with either the  $\beta_2$ AR expression vector or the control construct. The myocytes were  
 15 identified by GFP as described above. The total percentage of beating cells ( $\geq 1$  contraction/minute) was estimated visually from cotransfected GFP-positive myocytes ( $> 100$  cells/point). In addition, the percentage of myocytes that were beating faster than 60 beats per minute (bpm) was determined in identical fashion. Similar measurements of the percentages of both total and fast beating myocytes were  
 20 conducted at various concentrations of isoproterenol (control,  $10^{-5}$ ,  $10^{-4}$ , and  $10^{-3}$  M). Both the total percentage of beating myocytes and those with rates  $> 60$  bpm were used as a measure of automaticity.

The average rate of myocyte contraction was quantitated by motion detector under both baseline and  $10^{-3}$  M isoproterenol. Inverted microscopy with  
 25 epifluorescence filters for FITC<sub>(excitation 405 nm/emission 490 nm)</sub> and a video edge motion detector (Cresecent Electronics) were employed to determine the average rate of the contractions of GFP-positive myocytes. The statistical significance of the increased rate of myocyte contraction was determined by Student's *t*-Test analysis.

## RESULTS

### MYOCYTE CONTRACTILE RECRUITMENT

The percentage of cardiac myocytes that beat under baseline conditions was higher in the population of cells transfected (3-5% efficiency) with the  $\beta_2$ AR expression  
5 vector, as compared with the control LacZ expression vector. Figure 1A shows that under baseline conditions 67% of the  $\beta_2$ AR transfected myocytes exhibit spontaneous contractions as compared with 42% in the control LacZ transfected cells. The addition of increasing concentrations of isoproterenol elevated the percentage of control LacZ transfected myocytes to 69%, which is similar to the percentage of  $\beta_2$ AR transfected  
10 cells contracting at baseline. Moreover, the addition of isoproterenol failed to increase the number of contracting  $\beta_2$ AR transfected myocytes.

The percentage of myocytes with chronotropic rates greater than 60 bpm (fast beating cells) was higher in the  $\beta_2$ AR transfected myocytes as compared with the control transfected cells. Figure 1B shows that in the absence of isoproterenol 37% of  
15 the  $\beta_2$ AR transfected cells beat fast as compared with 15% of the control transfected cells. As compared to the total percentage of contracting cells, the number of fast beating cells increased in both the  $\beta_2$ AR and control populations with addition of isoproterenol. At  $10^{-3}$  M isoproterenol, the percentage of  $\beta_2$ AR transfected cells increased to 57%, which was not different from the response of the control transfected  
20 cells (54%).

### DIRECT RATE MEASUREMENTS

The average rate of contraction determined by motion detection was higher in the  $\beta_2$ AR transfected myocytes, as compared with control transfected cells under both baseline conditions and in the presence of  $10^{-3}$  M isoproterenol. Figure 2 reveals that  
25 under control conditions the average rate of contraction of  $\beta_2$ AR transfected myocytes was significantly higher as compared with control transfected cells (71 +/- 14 vs. 50 +/- 10 bpm;  $p < .001$ ). The average rate of contraction increased by a similar proportion in both populations with the addition of isoproterenol. In the presence of  $10^{-3}$  M



isoproterenol, the average rate of contraction of  $\beta_2$ AR transfected myocytes was significantly higher as compared with the control cells (98 +/- 26 vs. 75 +/- 18 bpm;  $p < .05$ ).

#### EXAMPLE 2: HEART TRANSPLANTATION AND DNA INJECTION

- 5 Neonatal B6D2F1 murine hearts were transplanted into the pinneas of adult mice as previously described (Fulmer, R. *et al.*, *American Journal of Anatomy*, 113:273-286 (1963); Rossi, M. A., *Am J Pathol.*, 141(1):183-91 (1992)). Briefly, recipient 6-week-old adult B6D2F1 mice were anesthetized with Avertin 2.5% (vol/vol). After cleaning the dorsum of the pinnea of the mouse ear with 70% ethanol,
- 10 an incision penetrating only the epidermis, 2-5 mm in length, was made with a scalpel transverse to the longitudinal axis of the ear, 3-4 mm distal to its implantation into the skull. A small pocket between the skin and cartilage was blunt dissected toward the tip of the ear with delicate curved forceps. The total donor neonatal heart was excised without the pericardial sac and inserted into the ear pocket. Gentle pressure with the
- 15 tips of the forceps was applied to the ear to express air from the pocket and facilitate the adherence between donor and recipient tissues. After 4 to 6 weeks post-transplantation, the transplanted hearts were assayed for visual pulsation and electrocardiographic activity. Visual pulsation of the transplanted tissue was observed in the anesthetized host mice under stereoscopic microscopy. Electrocardiograms (ECGs) of the
- 20 transplanted hearts were also recorded. Host mice were anesthetized and electrocardiogram limb leads were clipped to the ear surrounding the transplanted heart. ECGs were recorded with a Silogic EC-60 monitor (Silogic Design Limited). Approximately 80% of the transplanted hearts were observed to have visual pulsations and electrocardiographic activity.
- 25 Transplanted hearts with both visual pulsations and electrocardiographic activity were then employed in DNA injection experiments. After baseline ECGs were recorded from the transplanted hearts, expression vectors (prepared as described in Example 1) were injected into the atrium of the transplanted hearts similarly as previously described

in murine skeletal muscle injection (Wolff, J. A. *et al.*, *Science*, 247 (4949 Pt 1):1465-8 (1990)). Briefly, the  $\beta_2$ AR expression vector or the control construct (5 $\mu$ l of DNA (2 $\mu$ g/ml) in 20% sucrose, 2% Evans Blue, in PBS) were injected into the transplanted hearts with a 33-gauge needle. Electrocardiographic activity was recorded daily for up to 7 days following the injections. The statistical significance of increased heart rates was determined by a Student's *t*-Test analysis.

## RESULTS

### HEART TRANSPLANT DNA INJECTIONS

Injection of transplanted hearts with the  $\beta_2$ AR expression vector, as compared with control constructs generated an increased heart rate. ECGs recorded from the pinna surrounding the transplanted neonatal hearts demonstrate that  $\beta_2$ AR injected hearts exhibit faster cardiac rates, as compared to hearts prior to injection and to hearts injected with control constructs (Figure 3A). Recordings prior to injection revealed consistent electrocardiographic activity with an average heart rate of 180 +/- 20 bpm (Figure 3B). Two days after injection with the  $\beta_2$ AR expression vector, the heart rate increased to 220 +/- 20 bpm, which was significantly higher than the preinjection heart rate ( $p < .001$ ) or the heart rate observed with control constructs ( $p < .005$ ). The increased heart rate was sustained for 3 to 4 days after which the heart rate returned to baseline levels.

### EXAMPLE 3: INTRACARDIAC DNA INJECTION OF ENDOGENOUS MURINE HEARTS

The right atria of 6-week-old adult B6D2F1 murine hearts were injected with expression vectors (which were prepared as described in Example 1). Adult mice were anesthetized with avertin 2.5%, and a baseline ECG was recorded. The heart was exposed as previously described (Selge, H. *et al.*, *Angiology*, 11:398-407 (1960); Kitsis, R. N. *et al.*, *Proc Natl Acad Sci U S A.*, 88(10):4138-42 (1991)). Briefly, the mice were then intubated and mechanically ventilated with a rodent ventilator (Model 683,

Harvard Apparatus, Inc., South Natick, MA) with room air. A right anterolateral thoracotomy was then performed and the heart visualized. The  $\beta_2$ AR expression vector or the control construct was then introduced into the right atrial wall with a 30-gauge needle, as described above. The lungs were reexpanded and the chest closed in three  
5 layers with 4-0 silk sutures. The mice were then allowed to recover spontaneous respiration. Electrocardiographic activity was recorded daily for up to 7 days following the injections. The statistical significance of increased heart rate was determined by a Student's *t* Test analysis.

### $\beta_2$ AR IMMUNOSTAINING

10 Sections of injected hearts were immunostained for the human  $\beta_2$ AR as described above. Briefly,  $\beta_2$ AR and control expression vector injected transplanted or intact hearts were sectioned to 8- $\mu$ m sections and fixed with cold acetone for 10 min. The sections were then washed with PBS and blocked with 10% normal serum in PBS for 20 min. Samples were then incubated with rabbit-antihuman  $\beta_2$ AR polyclonal  
15 antibody (Santa Cruz Biotechnologies) at 1.0  $\mu$ g/ml as described above. Additionally, sections through the right lateral atrium, the peri-injection site, were scored for the frequency of specific immunostaining in hearts injected with the control and  $\beta_2$ AR constructs.

## RESULTS

### 20 INTRACARDIAC DNA INJECTIONS

Injection of the  $\beta_2$ AR expression vector increased the density of receptor in the right atrium of endogenous hearts. Immunostaining for the human  $\beta_2$ AR three days post injection revealed right atrial expression in the hearts injected with the  $\beta_2$ AR expression vector, but not in the hearts injected with control construct. Expression of  
25 the human  $\beta_2$ AR was detected in 81 +/- 13% of the myocytes in the peri-injection site of the targeted hearts, with no specific staining in the control injected atria. Surface ECGs recorded from adult mice demonstrate that hearts injected with the  $\beta_2$ AR expression

vector exhibit a higher rate of contraction as compared with hearts prior to injection as well as to the hearts injected with the control construct (Figure 4A). The average heart rate of the anesthetized adult mice was 370 +/- 20 bpm prior to injection (Figure 4B), similar to previously reported rates in resting and anesthetized mice (Milano, C. A. *et al.*, *Science*, 264(5158):582-6 (1994)). Two days after injection with the  $\beta_2$ AR expression vector, the heart rate increased to 550 +/- 42 bpm, which was significantly higher as compared to the preinjection heart rate ( $p < .01$ ), as well as to the control construct postinjection heart rate ( $p < .05$ ). The increased heart rate was sustained for 2 to 3 days after which time the heart rate returned to baseline levels.

#### 10 EXAMPLE 4: MOLECULAR ENHANCEMENT OF PORCINE CARDIAC CHRONOTROPY

The experiment outlined in this example was directed at developing an *in vivo* gene transfer technique to identify and study genes that can be employed to selectively upregulate heart rate and alter cardiac rhythm in the intact heart in a large animal model.

15 The Yorkshire pig was chosen for its anatomic and physiologic similarity to the human cardiovascular system, and because porcine models have been successfully employed in other gene therapy studies involving cardiac vasculature. Constructs encoding either the human  $\beta_2$  adrenergic receptor ( $\beta_2$ AR) or green fluorescent protein were injected into the right atrium of native Yorkshire pig hearts. Percutaneous electrophysiologic recording

20 catheters equipped with 33 g circular injection needle were positioned in the mid-lateral right atrium. At the site of the earliest atrial potential the circular injection needle was rotated into the myocardium, and the  $\beta_2$ AR (n = 6) or control plasmid constructs (n = 5) injected. The average atrial electrocardiogram to surface P wave interval at the injection site was similar in pigs injected with the  $\beta_2$ AR and control constructs (14 +/- 10 vs. 12 +/-

25 10 ms). The average PR interval and P wave axis were similar in the  $\beta_2$ AR- and control-animals, both at baseline and 48 hr post injection. Injection of the  $\beta_2$ AR construct significantly enhanced chronotropy, as compared to control injections. The average cycle length of the pig heart rate was 567 +/- 100 ms prior to injection. Two days after injection

with plasmid encoding the  $\beta_2$ AR the cycle length decreased to  $327 \pm 60$  ms, which was significantly faster as compared to the control cycle length  $488 \pm 130$  ms ( $p < .03$ ).

## PLASMID CONSTRUCTS

cDNA encoding the human  $\beta_2$ AR was the kind gift of Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham NC). A 2.25 kb Sal I-BamH I fragment, the human  $\beta_2$ AR SV40 cDNA was ligated into a Sal I-BamH I site 3' to the ( $\beta$  actin promoter) ( $\beta$ AP) in a pBR322 vector to generate pBR322- $\beta$ AP- $\beta_2$ AR-SV40. The plasmid construct encoding the humanized green fluorescent protein (GFP) with a CMV promoter element was purchased from Clontech, and served as a control vector. The injection vehicle was PBS with 20% sucrose and 2% Evans blue.

## INJECTION CATHETER

Electrophysiology recording catheters were custom designed and manufactured by Medtronic, Inc. The polyurethane-coated catheter was 7F in size and was supported with an 8F sheath. The distal end of the catheter was terminated with a  $3\frac{1}{2}$  turn 33 gauge corkscrew shaped needle allowing it to impale securely onto tissues to record local intracardiac electrograms. The proximal end of the catheter was terminated with a lure lock injection port allowing it to accept standard sized syringes. The total unit had 70  $\mu$ L of dead space).

## ELECTROPHYSIOLOGICALLY-GUIDED INTRACARDIAC INJECTION

Female Yorkshire pigs weighing 15-20 kg were initially anesthetized with intramuscular ketamine (10(g/Kg) and intubated. The animals were then given 2% isoflurane, and ventilated with a large animal ventilator (Hallowell model 2000). Heart rate, blood pressure and arterial oxygen saturation were monitored during the duration of the procedure. By sterile technique, the right femoral vein was exposed, cannulated, and an 8F sheath inserted. Under fluoroscopic guidance the 8F electrophysiologic injection catheter was introduced and advanced to the right atrium. Simultaneous 6 lead surface and

intracardiac electrocardiograms were recorded with a multichannel recorder (EVR PPG Biomedical). The A-P interval (ms), cycle length (ms), PR interval (ms), and P wave axis ( $^{\circ}$ ) were measured. At the site of earliest A wave the injection needle was rotated  $270^{\circ}$  into the atrial myocardium. The recombinant DNA constructs (200(L; 100 (g/mL), GFP alone  
 5 (n = 5) or  $\beta_2$ AR/GFP (5:1 M/M) samples (n = 6), were then injected into the atrial myocardium. The catheter was then disengaged and removed from the animal. The animal was observed from an additional 10 min and monitored for complications. The vascular sheath was then removed, the vein sutured, and the incision site closed. Anesthesia was then discontinued. After regaining spontaneous respirations that animals were placed in  
 10 individual pens. The animals were monitored on an hourly basis for the next three hours, and then daily until the termination of the experiments, 96 hr post injection.

#### SERIAL SURFACE ELECTROCARDIOGRAM RECORDINGS AND ANALYSIS

Serial surface electrocardiogram were recorded daily on all animals during the duration of the study. The pigs were anesthetized with ketamine as above. Simultaneous  
 15 6 lead surface electrocardiograms were recorded. The cycle length, PR interval, and P wave axis were measured. Statistical significance was determined by a Student's t-Test analysis.

#### $\beta_2$ AR IMMUNOSTAINING

At the termination of the experiments the animals were sacrificed and the  
 20 hearts explanted. The injection sites were harvested for sectioning and immunostaining.  $\beta_2$ AR and control expression vector injected atria were sectioned to 10 mm sections and fixed with cold methanol for 10 min. The sections were then washed with PBS and blocked with 10% normal serum in PBS for 20 min. Samples were then incubated with rabbit-anti-human  $\beta_2$ AR polyclonal antibody (Santa Cruz Biotechnologies) at 1.0  
 25 mg/mL for 1 hr. Samples were then incubated with the primary antibody at 1.0 mg/mL in PBS with 1% bovine serum albumin for one hr in a humid chamber at  $25^{\circ}\text{C}$ . The sample was then washed with PBS three times and then incubated with the secondary

donkey-anti-rabbit Cy3 polyclonal antibody (Jackson ImmunoResearch) at a 1:1000 dilution in PBS with 1% bovine serum albumin for one hr in a humid chamber at room temperature. The samples were washed with PBS three times and mounted with 90% glycerol in PBS. GFP expression was identified by employing epifluorescence filters for green fluorescence<sub>(excitation 405 nm/emission 490 nm)</sub>. Immunostaining for the human  $\beta_2$ AR was identified by employing epifluorescence filters for red fluorescence (excitation 488 nm/emission 540 nm).

## RESULTS

### ELECTROPHYSIOLOGICALLY-GUIDED INTRACARDIAC cDNA INJECTIONS

The animals were anesthetized, intubated, and venous access obtained as described above. The injection catheter was advanced to the right lateral atrium under fluoroscopic guidance. Simultaneous surface and intracardiac electro-cardiograms were recorded. The catheter was positioned at the site of the earliest atrial activity. The atrial potential at the injection sight was similar in both the pigs injected with the control (14 +/- 10 ms) and the  $\beta_2$ AR encoding constructs (12 +/- 10 ms). In addition, both the average PR interval and P wave axis on the surface ECG was similar for both groups prior to injection, Table 1. All the animals tolerated the procedure well.

### POST INJECTION ECG ANALYSIS

Serial surface ECGs recorded from the pigs after construct injection demonstrate that the average PR interval and P wave axis on the surface ECG was similar to the measurements prior to injection, Table 1.

Table 1

Electrocardiographic Measurements				
	Baseline		48 hr post	
	Control	$\beta_2$ AR	Control	$\beta_2$ AR
P wave axis ( $^{\circ}$ )	65 +/- 7	56 +/- 20	60 +/- 20	62 +/- 15
PR (ms)	85 +/- 10	92 +/- 9	86 +/- 8	86 +/- 9

5 The heart rate increased in the hearts injected with the  $\beta_2$ AR plasmid as compared to the control cycle lengths, Figure 2A. The cycle length of the pigs was 567 +/- 100 ms prior to injection. Two days after injection with plasmid encoding the  $\beta_2$ AR the cycle length decreased to 327 +/- 60 ms, which was significantly faster as compared to the control cycle length 488 +/- 130 ms ( $p < .03$ ), Figure 2B. The difference in cycle length after

10 control injection was not statistically significant ( $p > 0.3$ ). These changes correlated with a 49% increase in the average heart rate in the  $\beta_2$ AR injected pigs (183 +/- 28 vs. 122 +/- 25 bpm). The increased heart rate was sustained for 1 to 2 days after which the heart rate trended to baseline levels. All animals survived until the termination of the experiment. In summary, these studies demonstrate that the basal rate of the heart can

15 be enhanced by local delivery of exogenous genes. The present example demonstrates that local targeting of gene expression may be a feasible modality to regulate the cardiac pacemaking activity. In addition, the porcine model system also provide an experimental basis for developing future human clinical gene transfer protocols designed to upregulate heart rate and alter cardiac rhythm.

20 POST INJECTION IMMUNOSTAINING

Injection of the cDNA constructs lead to the expression of the encoded genes. Sections of the right atrial tissue at the site of injection revealed the presence of GFP. Immunostaining of the sections revealed a colocalization of the human  $\beta_2$ AR in the co-injected with the  $\beta_2$ AR-encoding constructs.



While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

## CLAIMS

What is claimed is:

1. A molecularly-mediated cardiac pacemaker construct comprising at least one gene that upregulates heart rate or alters cardiac rhythm suitable for localized gene expression in mammalian cardiac atrial tissue.
2. The cardiac pacemaker of Claim 1 wherein gene expression is localized to the sinoatrial node region of the right atria.
3. The cardiac pacemaker of Claim 2 wherein the gene is selected from the group consisting of: a  $\beta_2$ AR gene,  $\beta_1$ AR gene, and  $G_{\alpha s}$  gene.
4. The cardiac pacemaker of Claim 3 wherein the construct further comprises expression control elements.
5. The cardiac pacemaker of Claim 4 wherein the expression control element directs transient expression.
6. The cardiac pacemaker of Claim 4 wherein the expression control element directs stable expression.
7. The cardiac pacemaker of Claim 6 wherein the expression control element comprises an inducible promoter.
8. A cellular-based cardiac pacemaker comprising at least one cell transfected or transduced with at least one gene that upregulates heart rate or alters cardiac rhythm.

9. The cardiac pacemaker of Claim 5 wherein the gene is selected from the group consisting of: a  $\beta_2$ AR gene,  $\beta_1$ AR gene, and  $G_{\alpha s}$  gene.
10. The cardiac pacemaker of Claim 8 wherein the cell is selected from the group consisting of: a myoblast, a cardiomyocyte, a skeletal muscle myoblast, a fetal or embryonic cardiomyocyte and a cardiac-derived cell line.
11. The cardiac pacemaker of Claim 9 further wherein the cell is isogenic, allogenic, or xenogenic.
12. A method of regulating cardiac pacemaking activity in a mammal by introducing a biologic pacemaker according to Claims 1, 7, 8 or 11 into the sinoatrial node region of a mammalian heart.
13. The method of Claim 12 wherein the biological pacemaker is introduced by direct myocardial injection or endocardiac transfection or transduction.
14. A method of improving cardiac function in senescent heart tissue by introducing a biological pacemaker according to Claim 1, 7, 8 or 11 into an atrial chamber of a mammalian heart.
15. The method of Claim 14 wherein the biological pacemaker is introduced by direct myocardial injection or endocardiac transfection or transduction.
16. The method of Claim 15 wherein the biological pacemaker is a molecular-mediated cardiac pacemaker construct comprising a gene encoding a  $\beta_2$ -adrenergic receptor, and further wherein the method comprises *in vivo* administration of an adrenergic agonist.
17. The method of Claim 15 wherein the adrenergic agonist is isoproterenol.

18. The method of Claim 14 wherein the biological pacemaker is a cellular-based cardiac pacemaker construct comprising a transfected or transduced cell expressing a  $\beta$ 2-adrenergic receptor and further wherein the method comprises *in vivo* administration of an adrenergic agonist.
- 5 19. The method of Claim 18 wherein the adrenergic agonist is isoproterenol.
20. The method of Claim 18 wherein the transfected or transduced cell comprises at least one fetal or embryonic cardiomyocyte transfected with at least one gene that upregulates heart rate or alters cardiac rhythm.
- 10 21. A method of improving inotropic responsiveness in a mammal with a cardiac conductive tissue dysfunction by introducing a biologic pacemaker according to Claim 1, 6, 7 or 11 into the sinoatrial node region of a mammalian heart.
- ~~22.~~ A method of treating a mammal suffering from a heart attack or transient depression of heart rate by delivering to the mammal a transient molecularly-mediated cardiac pacemaker.
- 15 ~~23.~~ A method of permanently regulating cardiac pacemaking activity in a mammal by introducing a cellular-based cardiac pacemaker comprising at least one fetal or embryonic cardiomyocyte transfected or transduced with at least one gene that upregulates heart rate or alters cardiac rhythm.
- 20 ~~24.~~ A method of permanently regulating cardiac pacemaking activity in a mammal by introducing a molecularly-mediated cardiac pacemaker construct comprising at least one gene that upregulates heart rate or alters cardiac

rhythm suitable for localized stable gene expression in mammalian cardiac atrial tissue.

- 25. The method of Claim 24 wherein the molecularly-mediated cardiac pacemaker construct comprises an inducible promoter.

0050.1609-002

## ENHANCEMENT OF CARDIAC CHRONOTROPY

## ABSTRACT OF THE DISCLOSURE

Molecularly-mediated and cellular-based  $\beta$ -adrenergic receptor-dependent biological pacemakers are disclosed. Methods of using these compositions to improve cardiac chronotropic responsiveness by upregulating heart rate and altering cardiac rhythm are also disclosed.

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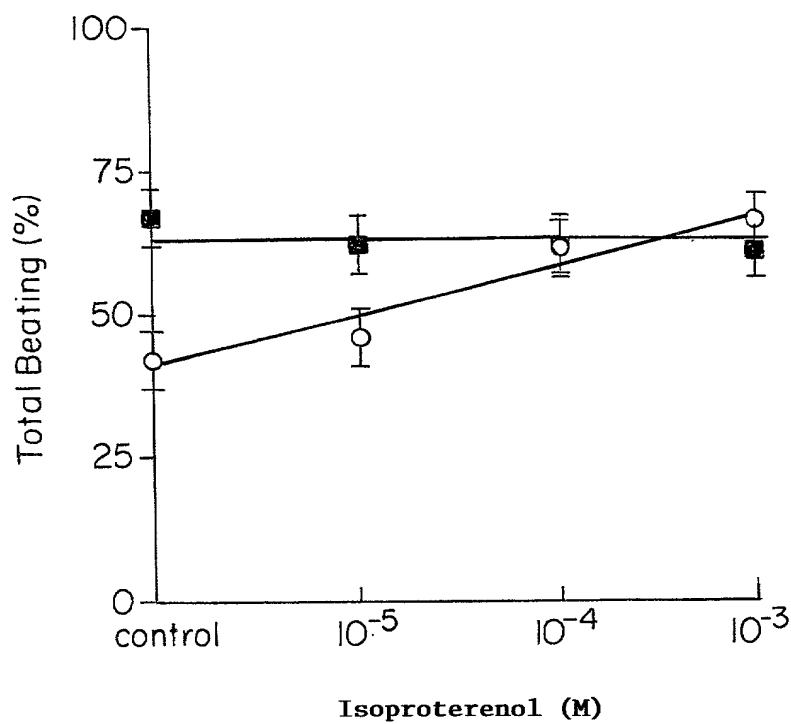


FIG. 1A

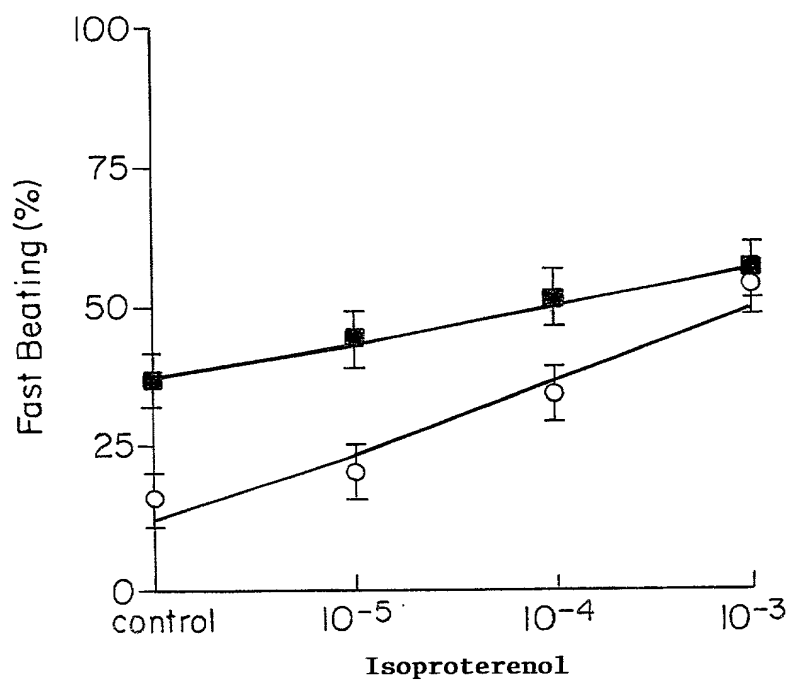


FIG. 1B

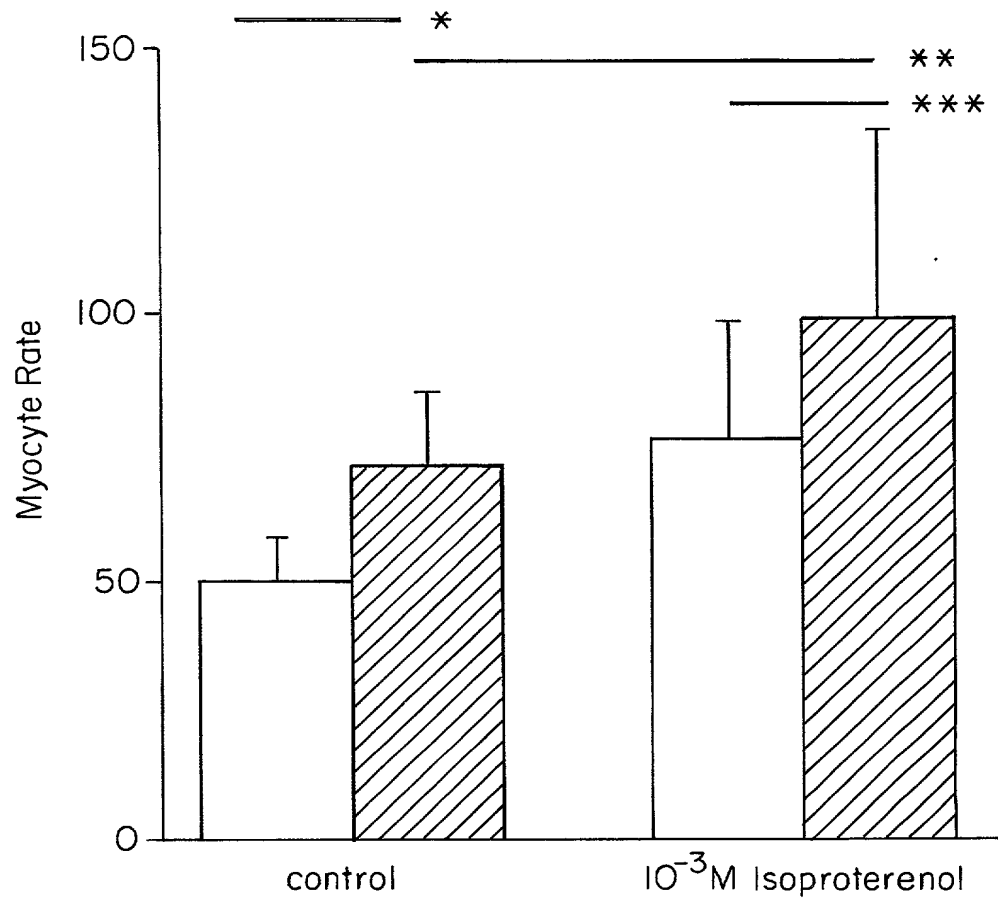


FIG. 2



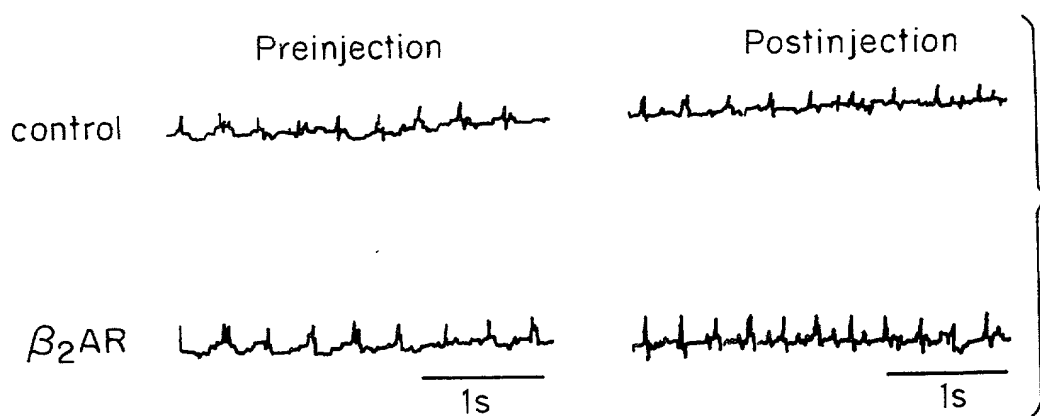


FIG. 3A

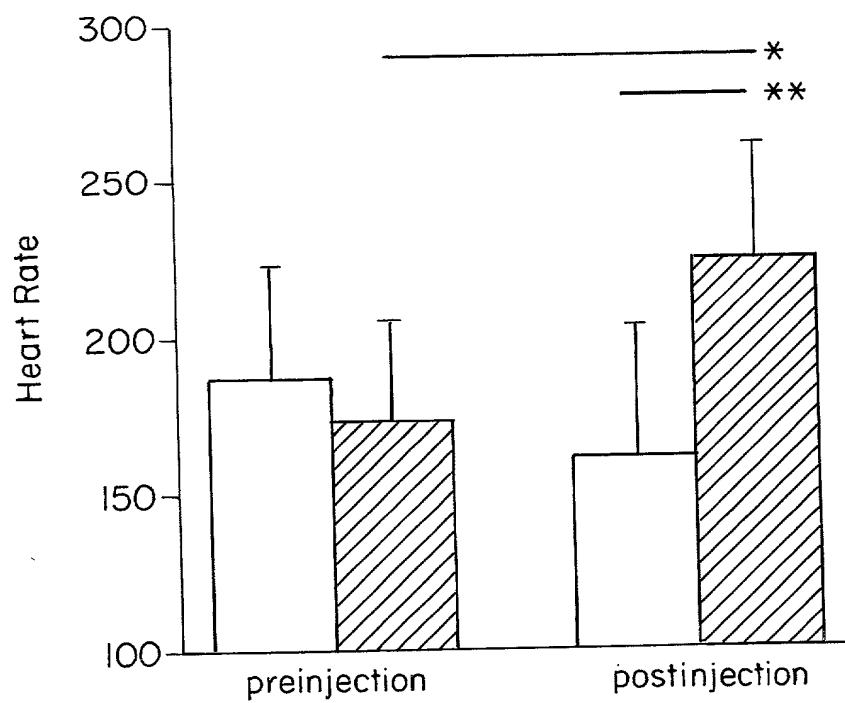


FIG. 3B

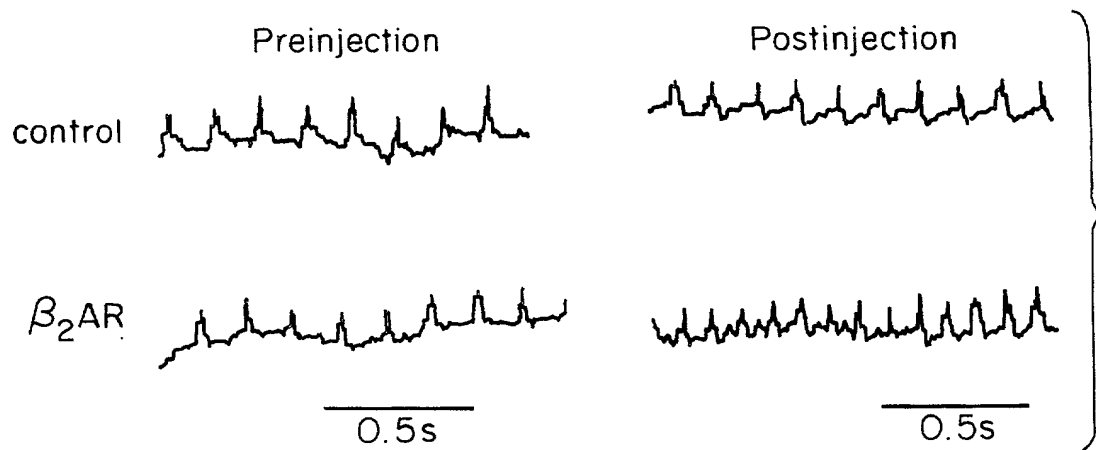


FIG. 4A

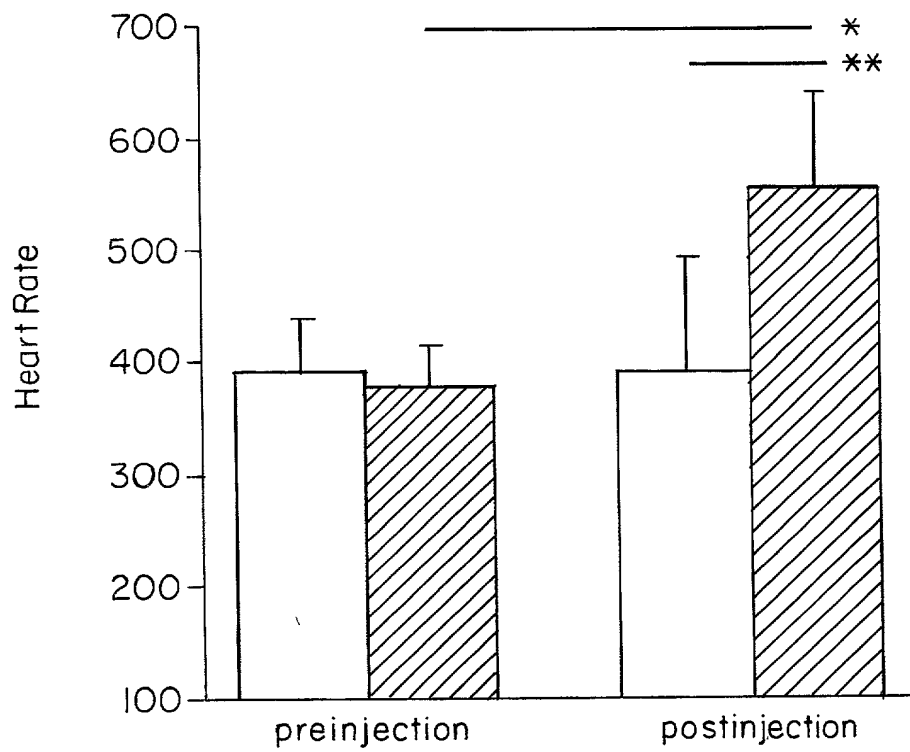


FIG. 4B

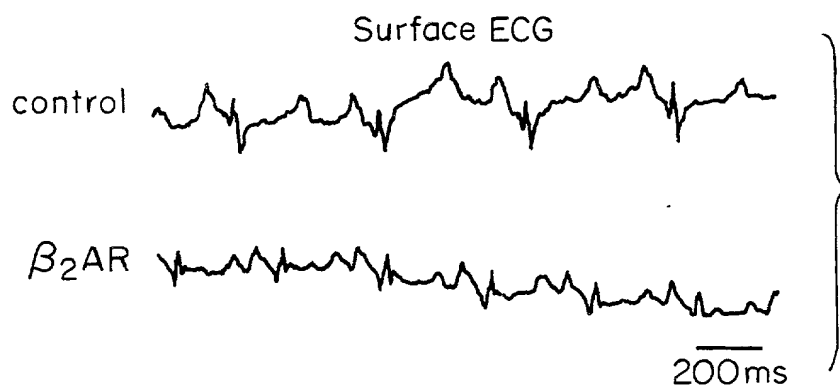


FIG. 5A

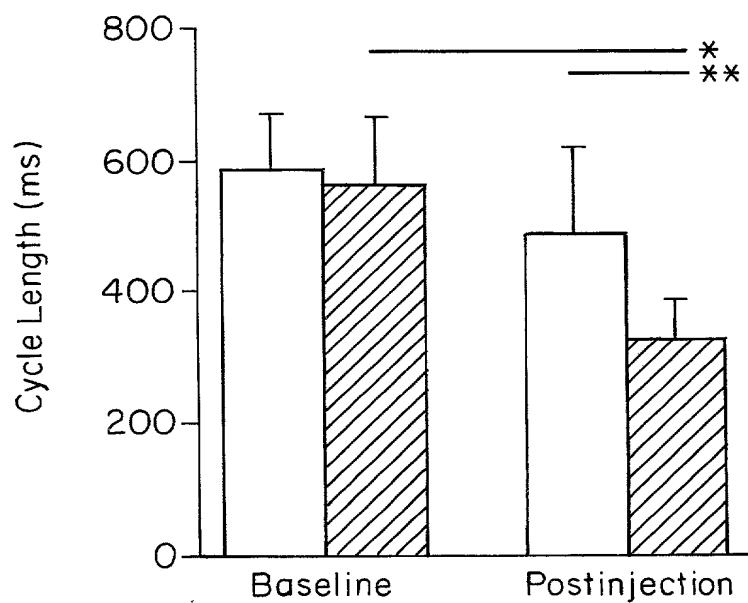


FIG. 5B